AWARD NUMBER: W81XWH-12-1-0302

TITLE: White Matter Glial Pathology in Autism

PRINCIPAL INVESTIGATOR: Gregory A. Ordway, Ph.D.

CONTRACTING ORGANIZATION: East Tennessee State University
Johnson City, TN 37614

REPORT DATE: September 2014

TYPE OF REPORT: Annual Report

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

# REPORT DOCUMENTATION PAGE

*Form Approved*
*OMB No. 0704-0188*



| 1. REPORT DATE | 2. REPORT TYPE | 3. DATES COVERED |
|---|---|---|
| September 2014 | Annual | 1 Sep 2013 - 31 Aug 2014 |

**4. TITLE AND SUBTITLE**
White Matter Glial Pathology in Autism

**5a. CONTRACT NUMBER**

**5b. GRANT NUMBER**
W81XWH-12-1-0302

**5c. PROGRAM ELEMENT NUMBER**

**6. AUTHOR(S)**
Gregory A. Ordway, Ph.D.; Michelle J. Chandley, Ph.D.; Jessica D. Crawford, Ph.D.

E-Mail: Ordway@etsu.edu

**5d. PROJECT NUMBER**

**5e. TASK NUMBER**

**5f. WORK UNIT NUMBER**

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**
East Tennessee State University
807 University Pkwy
Johnson City, TN 37614-6500

**8. PERFORMING ORGANIZATION REPORT NUMBER**

**9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

**10. SPONSOR/MONITOR'S ACRONYM(S)**

**11. SPONSOR/MONITOR'S REPORT NUMBER(S)**

**12. DISTRIBUTION / AVAILABILITY STATEMENT**

Approved for Public Release; Distribution Unlimited

**13. SUPPLEMENTARY NOTES**

**14. ABSTRACT**
Methods used to directly study the autism brain include brain imaging in living patients and pathology studies using postmortem brain tissues from deceased autism spectrum disorder (ASD) donors. These methods typically focus on brain regions as a whole with little regard to the underlying cellular complexity. While informative, these approaches do not provide information about the specific brain cells affected and also have not been successful in revealing the underlying cause of autism. This project uses innovative methods and a novel approach to investigate the pathology of the brain autism spectrum disorder (ASD). This research employed laser capture microdissection to isolate specific cell populations from carefully defined and specific brain regions from ASD and typically developing control brains. These cells were used to interrogate gene expression abnormalities that may underlie biological mechanisms that contribute behavioral abnormalities of ASD. By examining the ASD brain at the level of its most basic component, the cell, we seek to reveal a potentially unifying cellular pathology of the ASD brain that could be used for the development of therapeutic alternatives for ASD patients.

**15. SUBJECT TERMS**
laser capture microdissection, transcriptional analysis, RNA-Seq, autism spectrum disorder, postmortem human brain tissue

| 16. SECURITY CLASSIFICATION OF: | | | 17. LIMITATION OF ABSTRACT | 18. NUMBER OF PAGES | 19a. NAME OF RESPONSIBLE PERSON |
|---|---|---|---|---|---|
| a. REPORT | b. ABSTRACT | c. THIS PAGE | | | USAMRMC |
| Unclassified | Unclassified | Unclassified | Unclassified | 14 | 19b. TELEPHONE NUMBER *(include area code)* |

# Table of Contents

# INTRODUCTION

Within the autism spectrum disorder (ASD) research field to date, molecular pathology studies used brain samples that contain multiple cell types. The results of these studies have been insufficient to formulate a theoretical etiology of the disease. ASD is a spectrum of disorders with patients exhibiting vast differences in symptom presentation. By using an approach targeted at molecular pathology within a single cell population, a common cellular dysfunction might be found that could unify our conceptualization of ASD brain pathology throughout the spectrum. Identification of key cellular abnormalities could result in the development of novel targeted treatments for ASD. In this project, LCM is used to obtain clusters of multiple cell types and separately, selected cell populations. Using these samples, we are attempting to develop experimental protocols to permit gene expression profiling using RNA-Seq technology. Ultimately, we hoped to identify specific gene expression abnormalities in specific cell populations to further our understanding of ASD pathology. This type of analysis has the potential to bring light to unanswered questions of ASD pathology, and also to establish a powerful method to investigate the contributory roles of different brain cell types in neurological diseases.

# KEYWORDS

laser capture microdissection, transcriptional analysis, postmortem human brain tissue, RNA-Seq, autism spectrum disorder (ASD)

# ACCOMPLISHMENTS

**What were the major goals of the project?**

The overall goal of this project was to use transcriptional analysis of single cell populations to gain a better understanding of ASD brain pathology. This goal was divided into two tasks briefly described below.

- Task 1 involved the laser capture and preparation of postmortem brain tissue samples from the anterior cingulate cortex for analysis using RNA-Seq. This analysis included both sequencing and bioinformatics to determine genes/pathways of interest. Gene expression changes found to be different between control and ASD samples would be confirmed using end-point PCR methods. (To be completed by the end of 2014)
- Task 2 further examined the RNA-Seq finding by determining if the gene expression changes found in the anterior cingulate cortex were also present in the prefrontal cortex. This task involved the laser capture of brain tissue samples for end-point PCR analysis for the genes chosen in Task 1. (To be completed by August 2015)

**What was accomplished under these goals?**

Task 1

*Methods*

Frozen tissue blocks containing BA24 from six ASD donors and eight typically developed control donors (Table 1) were obtained from Autism Tissue Program, Harvard Brain Tissue Resource Center (Belmont, MA) and NICHD Brain and Tissue Bank for Developmental Disorders (Baltimore, MD). Additional subject samples were prepared but excluded from analysis at various stages of preparation due to poor sample quality factors such as low RIN or insufficient sequencing reads. Superficial white matter was laser captured from BA24 brain sections (10 μm thickness) mounted on PEN membrane glass slides (Life Technologies, Grand Island, NY). Superficial white matter was defined as the white matter area directly adjacent to gray matter and within 3 mm of the white/gray matter border area. Multiple large circular areas were captured for each sample. Pyramidal neurons and white matter astrocytes were stained and captured from BA24 cortical layer 3. Neurons were visualized by staining frozen 10 μm thick sections with the Histogene staining kit (Life Technologies; Grand

Island, NY) according to manufacturer's instructions. Astrocytes were identified using a modified glial fibrillary acidic protein (GFAP) rapid immunohistochemistry protocol as previously described[1,2].

RNA was isolated from the captured samples using PicoPure RNA Isolation Kit (Life Technologies, Grand Island, NY) with the additional RNase-free DNase kit (Qiagen, Valencia, CA) step outlined in the manufacturer's protocol. The Ovation Single Cell RNA-Seq System (NuGEN, San Carlos, CA) was used to generate RNA-Seq libraries from isolated RNA. Extensive quality control was performed. Pooled libraries were sent to David H. Murdock Research Institute for sequencing. The HiSeq2500 instrument (Illumina, San Diego, CA) was used for 100 base paired reads with indexing sequencing using the instrument's high output sequencing run. Following sequencing, base calling was performed with CASAVA (v1.8.2) (Illumina, San Diego, CA). Filtering and trimming of reads consisted of removal of Illumina Adapter Library and trimming in the CLC Genomics Workbench 7.0.4 (Qiagen, Valencia, CA). Reads were then aligned to the human genome (latest version, hg38/GRCh38, assembled on December 2013, annotations updated in June 2014) using CLC Genomics Workbench 7.0.4 using the CLC's RNA-Seq package. The Baggerly Beta-binomial test [3] was performed for group comparisons using the control donors as the reference. A false discovery rate (FDR) correction was used to further correct p-values achieved using the above proportion-based tests. We used both paired and unpaired statistical comparisons of control and autism gene expressions for these preliminary data understanding that with the small sample size, neither approach is likely to produce data with high statistical confidence.

For PCR confirmation of RNA-Seq data, RNA was isolated from the captured samples using PicoPure RNA Isolation Kit (Life Technologies, Grand Island, NY) with the additional RNase-free DNase kit (Qiagen, Valencia, CA) step outlined in the manufacturer's protocol. RNA samples were reverse transcribed into cDNA using the Superscript III kit (Life Technologies; Grand Island, NY) that contained oligodT and random hexamer primers. Gene specific primers were purchased from a vendor (Qiagen; Valencia, CA). To quantify transcripts, endpoint PCR was used for RNA isolated from laser captured cells as previously described [2,4]. Endpoint PCR data was computed as relative values generated from the ratios of amounts of target gene expression to a reference gene. Afterwards, endpoint PCR data were analyzed by the paired Student's t-test.

**Table 1.** Subject demographic information.

| ID | Age | Gender | RIN[a] | PMI (hours)[b] | Toxicology | Sample |
|---|---|---|---|---|---|---|
| **Controls** | | | | | | |
| AN14757 | 24 | M | 7.8 | 21.33 | No drugs reported | WM[d], Neuron |
| AN07444 | 17 | M | 7.5 | 30.75 | Sertraline | WM |
| 5408 | 6 | M | 7 | 16 | No drugs reported | WM, Neuron |
| 4848 | 16 | M | 7.6 | 15 | No drugs reported | Neuron |
| 5342 | 22 | M | 8.1 | 14 | No drugs reported | WM |
| 5079 | 33 | M | 7.3 | 16 | Ethanol | WM |
| M3231M | 37 | M | 7.4 | 24 | No drugs reported | WM, Neuron |
| 4337 | 8 | M | 8.4 | 16 | No drugs reported | WM |
| **MEAN** | **20.38** | | **7.64** | **19.14** | | |
| **SEM** | **3.88** | | **0.16** | **2.05** | | |
| | | | | | | |
| **ASD** | | | | | | |
| AN04166 | 24 | M | 8.1 | 18.51 | No drugs reported | WM, Neuron |
| AN02987 | 15 | M | 6.5 | 30.83 | No drugs reported | WM |
| 5144 | 7 | M | 8 | 3 | No drugs reported | WM, Neuron |
| 5302 | 16 | M | 6.6 | 20 | Risperidone, Fluvoxamine, Clonidine, Insulin | Neuron |
| 5027 | 37 | M | 7.7 | 26 | Risperidone, Fluvoxamine | WM, Neuron, Astrocyte |
| 4721 | 8 | M | 6.1 | 16 | No drugs reported | WM |
| **MEAN** | **17.83** | | **7.17** | **19.06** | | |
| **SEM** | **4.59** | | **0.35** | **3.90** | | |
| **P value[g]** | **0.68** | | **0.26** | **0.99** | | |

[a] RNA integrity number (index of RNA quality)
[b] Postmortem interval
[c] Results of a two-tailed independent t-test comparing control and ASD groups.
[d] White matter

## *Results*

### *Sequencing Quality Based on Phred Score*

The first step in the analysis of sequencing is base-calling. This process consists of taking the multiple single nucleotide reads and composing the sequence of the fragment clusters. Using the Phred scoring methods, a cut-off score of around 30 is considered an acceptable quality for base-calling. A Phred score of 30 translates to a 1 in 1000 probability of an incorrect base-call or a 99.9% accuracy in sequence detection [5,6]. The PHRED score for all samples (white matter, neuron, and astrocyte preparations) exceeded this cut-off by reaching an average score of 35 to 40. A score of 40 translates to a probability of 1 in 10,000 incorrect base-call or a 99.99% accuracy in detection.

### *Mapping and Alignment*

*Mapping Percent and Total Reads.* The three types of sample preparations, white matter containing mixed populations of cells, pyramidal neurons, and GFAP-positive astrocytes, were compared to determine if there was a difference in mapping percentages based on sample type. There was no significant effect of sample type on the type of read produced by the samples (Figure 1). White matter and neuron samples were further analyzed to determine if there was a difference in the mapping

between control and ASD subject samples. No difference was found between control and ASD samples for white matter (Figure 2A, p = 0.64) or neuron (Figure 2B, p = 0.63) preparations.

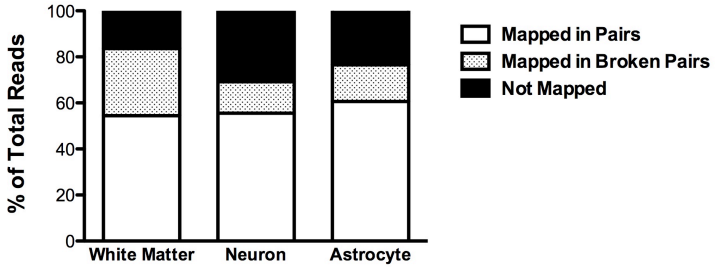


**Figure 1.** Division of read types of the white matter, neuron, and astrocyte RNA-Seq samples. The percent of reads mapped in pairs (white bars), mapped in broken pairs (shaded bars), and reads not mapped (black bars) were plotted as a percent of total reads for all samples.

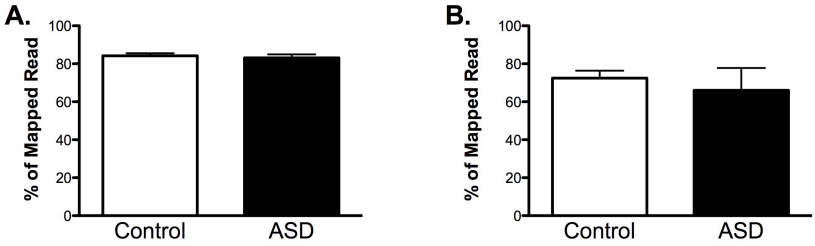


**Figure 2.** Comparison of read mapping between control and ASD samples for white matter (A) and neurons (B). The percentage of total mapped reads (mapped in pairs and broken pairs) of total reads was plotted. No significant difference was found between control and ASD samples for white matter or neuron samples.

*Paired Read Mapping.* For reads that were mapped as pairs, an analysis was done to investigate where those reads aligned (Figure 3). Approximately 75% of all mapped paired reads aligned to intron regions for all sample preparations. This was also the case when examining the alignment pattern between control and ASD samples for white matter (Figure 4A) and neuron (Figure 4B) sample preparations. To ensure that observed intronic read mapping was in agreement with previously reported brain sample RNA-Seq data, the percentage of intron reads was reported (Table 2) for known high intronic genes (Ameur et al., 2011). Each gene examined had a 52 to 100% intron mapping percentage.

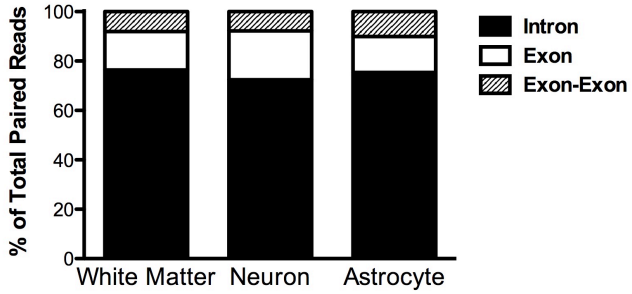


**Figure 3.** Comparison of read type for paired reads in white matter, neuron, and astrocyte samples. The percent of exon (white bar), exon-exon (shaded bar), and intron (black bar) reads of total paired reads were plotted.

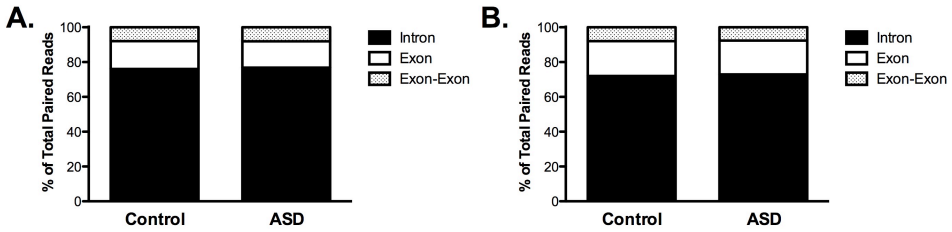


**Figure 4.** Comparison of read type for paired reads in control and ASD white matter (A) and neuron (B) samples. The percent of exon (white bar), exon-exon (shaded bar), and intron (black bar) reads of total paired reads were plotted for white matter and neuron control and ASD samples.

**Table 2.** Percentage of Intron Reads for Known High Intronic Genes.
Ameur et al. [7] produced a ranking of genes in the adult and fetal brain that produce the highest amount of intronic reads using RNA-Seq. The top genes from those lists were examined in our data. The table below contains the mean percentage of intron reads for each of the genes listed for all white matter, neurons and astrocyte samples. For white matter and neurons samples, the mean percentage of intron reads is given for control and ASD subjects separately.

| Gene Name | White Matter | | Neurons | | Astrocyte |
|---|---|---|---|---|---|
| | Control | ASD | Control | ASD | |
| **PCDH9** | 85.69% | 87.56% | 91.38% | 92.20% | 82.50% |
| **PCDH7** | 93.29% | 83.84% | 85.18% | 87.38% | 71.70% |
| **QKI** | 57.90% | 62.96% | 66.95% | 52.15% | 69.90% |
| **NRXN1** | 89.63% | 92.92% | 86.05% | 88.33% | 96.60% |
| **KCNC2** | 72.24% | 68.44% | 88.93% | 56.78% | 100.00% |
| **PID1** | 99.84% | 99.98% | 99.70% | 99.70% | 100.00% |
| **KLF7** | 70.51% | 69.28% | 64.50% | 70.98% | 60.90% |

*Differentially Expressed Genes.* A list of differentially expressed genes (DEGs) comparing control and ASD donor samples (Figure 5) was produced for white matter and neuron samples preparations. The expression level of these genes was determined by the RPKM (reads per kilobase per million mapped reads) of exon mapping reads only. Two genes, *STX8* and *SSR3*, were selected for confirmation of DEGs in the BA24 pyramidal neuron samples. The expression of the two genes was not found to be different comparing the same control and ASD donors as was used for the RNA-Seq experiment (Figure 6).

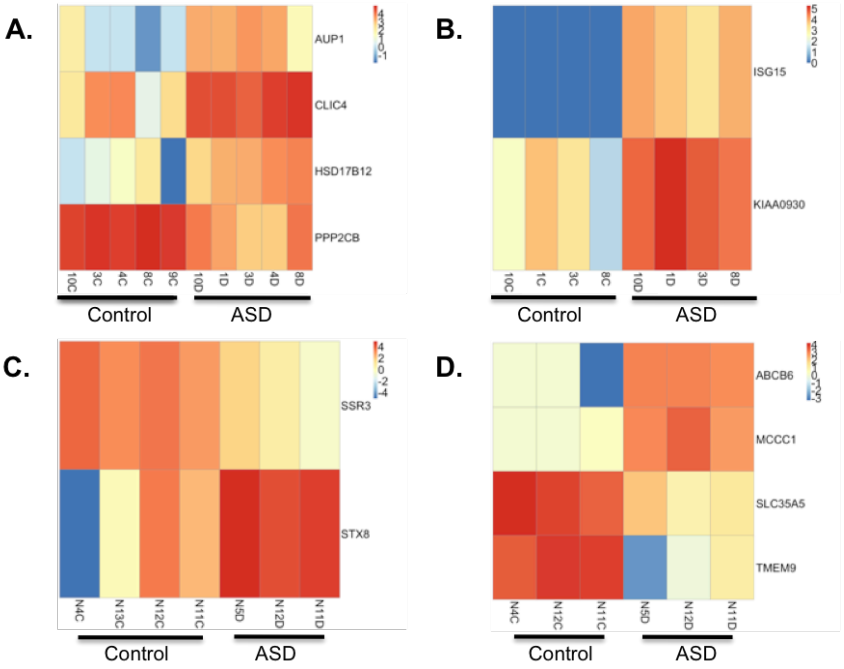


**Figure 5.** Differentially expressed genes in white matter and neuron samples. These heat maps represent the log2 transformed normalized expression value (RPKM) for each sample using a Baggerly beta-nominal test analysis. (A) white matter unpaired analysis (B) white matter paired analysis (C) neuron unpaired analysis (D) neuron paired analysis

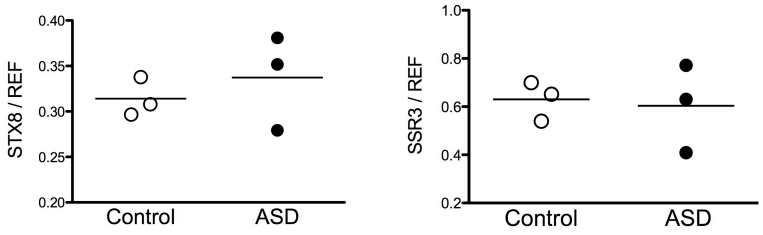


**Figure 6.** Levels of expression of *STX8* and *SSR3* in pyramidal neurons of BA24.
Gene expression was measured in laser captured BA24 pyramidal neurons from typically developing control donors (open symbols) and ASD donors (closed symbols). Gene expression levels are normalized to *GAPDH* expression levels. Mean values are noted by horizontal lines. No statistically significant differences were observed.

*Abnormalities in Intron Mapping in ASD.* Because the majority of the mapped pair reads were aligned to intron regions, we examined the possibility that the percentage of intron reads might be predictive of an underlying pathological process in ASD. To start with, two genes found to be differentially expressed between control and ASD subjects using exon mapped reads were compared at the level of mapped introns. From RNA-Seq data of control and ASD donors, we compared the reads mapped to *STX8* and *HSD17B12* introns as a fraction of the total reads. Those genes were chosen to have a comparison of how intron reads mapped for one gene with altered exon expression in neurons and one with altered exon expression in white matter. The fraction of reads mapped to *STX8* introns was significantly lower in ASD donors in neurons but not white matter. In contrast, the fraction of *HSD17B12* reads mapping to intron regions was lower in white matter but not neurons comparing control and ASD donors (Figure 7).

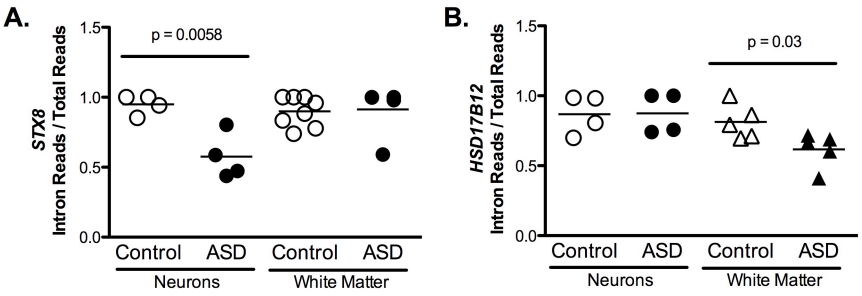


**Figure 7.** Comparison of intron reads for *STX8* and *HSD17B12* between control and ASD subject samples for neuron and white matter sample preparations. The ratio of intron reads to total reads for each gene is plotted for control (open symbols) and ASD (closed symbols) subjects for neurons and white matter samples. Statistical significance is noted in graph.

*Discussion*

One of the goals of this project was to establish the feasibility of using RNA-Seq to examine gene expression in brains cells captured by LCM. Three different LCM sampling methods were performed to determine which was suitable for this type of analysis. Overall, the quality of the sequencing data was high with a PHRED score that exceeded 35 and over 75% of all reads mapping to the reference genome for all sample preparation types (white matter, neuron, and astrocyte). There were also no observable differences between samples from control and ASD subject in terms of the quality of the RNA-Seq data generated. Once the ability to sequence the prepared libraries and map those reads to a reference genome was confirmed, the next step was to determine what the mapped reads represented.

Another goal of this project was to utilize LCM/RNA-Seq to compare the gene expression profiles of brain neurons and glia from typically developing control and ASD donors. One obvious and major problem with this part of the project was a small sample size, and hence the low power of the statistical analyses of the comparison of the two study groups. During the sample preparation stage, there were more subject samples than those reflected in the final analysis above. However, due to various reasons such as poor RIN quality, low mapping rate, and low RNA-Seq library yield, samples from some subjects were eliminated prior to RNA-Seq analysis. For the few control-ASD pairs that we were able to analyze, we generated a list of differentially expressed genes for the RNA-Seq data for neuron and white matter samples (Figure 5). However, an attempt to replicate some of the RNA-Seq data showing DEGs using PCR failed, with only two of the three pairs matched the RNA-Seq data by showing greater levels of *STX8* expression in ASD subjects compared to the controls using PCR confirmation (Figure 6). The small sample size could have been a cause for the inability to confirm the RNA-Seq finding with PCR. We are currently working to prepare more RNA-Seq libraries from additional paired subjects for analysis. For now, we assume that our inability to fully confirm existing RNA-Seq data is related to the small group sizes.

Within all the sample preparation types, a high percentage of intron mapping was observed. This is consistent with other RNA-Seq studies performed on brain tissue samples [7,8]. The high amount of intron reads does not affect our ability to determine differential expressed genes since the RPKM or expression value is determined by exon mapping reads only. However, these intron reads and the differences therein between control and ASD samples suggest that other forms of transcriptional regulation in the brain may differentiate ASD from control subjects. There is evidence that genes involved in neuronal plasticity and synaptic regulation are stored in an unprocessed RNA form within cell [7]. Based on the results shown in Figure 7, the ratio of intron reads to total reads is significantly different comparing control and ASD samples. This could reflect that the regulatory control in processing pre-mRNA to fully mature mRNA could be altered in ASD. This hypothesis will need to be confirmed using intron specific primers, and we are preparing to do this presently.

One of the disadvantages to using LCM collected samples is the small amount of material that can be obtained. When coupling LCM with the use of postmortem brain tissue, restrictions such as cost, time, and limited availability of tissue does not allow for the collection of sufficient amounts of input materials needed for many downstream applications. Due to the limited amount of sample that can be

obtained from LCM, sample amplification is an unavoidable preparation step in the transcriptional analysis. The amplification step in sample preparation is a sensitive process that could introduce experimental artifact if not performed correctly or tailored to specific biological samples. Experiments were performed using both commercially available kits and LCM specialized protocols to determine the most effective and reliable method for sample amplification. The first strand synthesis step of RNA amplification is crucial since it creates the basic template for amplification. Most methods are based on a 3' bias selection for this step of amplification. This selection technique is beneficial for reducing other RNA species such as rRNA and tRNA from the sample prior to mass amplification. This selection reduces potential bias toward more abundant RNAs since mRNA and non-coding RNA are significantly outnumbered by other RNA species in the samples. For samples collected from LCM, this 3' bias could reduce fidelity in amplification. Using frozen human tissue for these studies means that optimal RNA quality is never achieved because of decay that occurs during the brain collection process. A 3' bias selection could inadvertently exclude mRNAs that are susceptible to 3' degradation. Unlike other kits available on the market, the NuGEN amplification kits create a first strand using 3' and random primers giving better transcriptome coverage and reducing potential bias from degradation effects. This feature makes the kits ideal for LCM samples based on its tolerance for less than optimal RNA integrity and a small RNA input requirement of 100 picograms. However, the lack of ribosomal depletion or mRNA selection could have inadvertently biased our samples to pre-mRNA or intron containing transcripts. Intron spanning regions are larger than exon regions and would be more abundantly represented in the samples. It is still unclear if our samples produced enough exon based reads to truly reflect transcriptional changes at the mature mRNA level. Our samples might illustrate the regulation occurring between transcription and the finally mRNA product. More confirmation of the RNA-Seq data and more samples will be needed in order to fully answer this question; this work is currently underway.

*Conclusion*

When developing this method, efforts were made to circumvent the potential pitfalls associated with analyzing LCM samples. We were able to control for the limitations of these samples by selecting protocols suitable for sample type and putting in controls for the biological variances of human studies. There is still more work to be done to produce a full-scale analysis of transcription regulation underlying ASD brain pathology. In order to achieve that goal, there must first be a characterization of the type of transcriptional regulation reflected in our data whether it is exon- and/or intron-based. The data presented above suggest potential regulation at the pre-mRNA level in addition to that at the mature mRNA level. Further examination of intron changes could reveal yet unknown transcriptional dysregulation associated with ASD brain pathology.

**What opportunities for training and professional development has the project provided?**

Training

Work towards the completion of this project provided a training experience for the graduate student in the PI's lab. The work completed on the project thus far was included in this student's dissertation to fulfill the requirement for her Ph.D. in Biomedical Sciences (see Products section for more details).

Professional Development

The travel expenses for this grant were used to send the key laboratory scientist, Dr. Michelle Chandley, and the PI's trainee, Jessica Crawford, to the 2014 International Meeting for Autism Research held in Atlanta, GA. This meeting was hosted by the International Society for Autism Research and other nonprofit agencies that both support and fund autism research. This conference allowed these individuals to meet experts, identify future collaborative projects, and gain exposure to other projects happening in the field.

**How were the results disseminated to communities of interest?**

Nothing to report.

**What do you plan to do during the next reporting period to accomplish the goals?**

Additional ASD and control samples have been processed and submitted for RNA sequencing and bioinformatics analysis. While these samples are undergoing analysis, laser capture microdissection will be completed for all remaining subjects for PCR confirmation of RNA-Seq data. Following sample capture and preparation, PCR confirmation will be completed for both brain areas.

# IMPACT

**What was the impact on the development of the principal discipline(s) of the project? What was the impact on other disciplines?**

Ginsberg et al[9] has shown significant disparities between gene expression differences found using homogenate brain samples versus laser captured single cell populations from the same tissues. The Ginsberg study used microarrays to examine the brains of control and Alzheimer's disease (AD) subjects. Microarray data from CA1 captured cell populations displayed a dissimilar gene expression profile compared to the data obtained from hippocampal homogenate samples. Genes found to be essential for the dysfunctional of CA1 neurons in AD were not found significantly altered in the homogenate sample. This finding demonstrates the need to focus gene expression profiling on single cell populations in order to achieve a clear picture of the factors and pathways involved in cellular pathology. Within the ASD research field, all molecular pathology studies used brain samples that contain multiple cell types. To date, the results of these studies have been insufficient to formulate even a theoretical etiology of the disease. In order to more clearly define ASD pathology, greater focus must be placed on pathology at the cellular level. ASD is a spectrum of disorders with patients exhibiting vast differences in symptom presentation. By using an approach targeted at molecular pathology within a single cell population, a common cellular dysfunction could be found that could unify our conceptualization of ASD brain pathology throughout the spectrum. Identification of key cellular abnormalities could result in the development of novel targeted treatments for ASD. In this project, LCM is used to obtain selected brain tissue and cell populations that undergo gene expression profiling using RNA-Seq technology. Information collected from these experiments could identify the potential contributing roles of specific cell types in ASD pathology. The proposed research is interrogating only a part of the data generated by RNA-Seq, notably levels of transcripts. RNA-Seq also provides in-depth transcriptome analysis that cannot be achieved using methods such as microarrays, such as splicing variants, transcriptional start points, and rare RNA isoforms. These data will be available for mining in future studies, although it is likely that additional samples will be needed to enhance the power of an analysis of sequence. RNA-Seq permits researchers to explore all levels of transcriptional regulation in the cell [10–12]. To date, this method has not been utilized to explore molecular pathologies in selected cell populations. This results of this project will not only bring light to unanswered questions of ASD pathology, but will also establish a method that has not previously been used to investigate the contributory roles of specific brain cell pathology in neurological diseases.

**What was the impact on technology transfer?**

Nothing to report.

**What was the impact on society beyond science and technology?**

Nothing to report.

# CHANGES/PROBLEMS

**Changes in approach and reasons for change**

Method for Transcriptional Analysis

As outlined in the project summary submitted July 2014, a change has been made in the method of analysis for transcriptomics. RNA-Seq technology is being employed in place of microarrays. The

use of RNA-Seq technology allows for an increase in the amount of transcriptional data produced as well as an expansion in the downstream options for analysis. Overall RNA-Seq better suits our sample type and produces a more detailed picture of transcriptional regulation in ASD.

Additional Sample Types Added to Analysis

This project was focused on the cellular basis of ASD brain pathology. In order to determine the the feasibility of the LCM/RNA-Seq approach for human brain pathology research, we submitted multiple sample types for RNA-Seq analysis as discussed in the Accomplishments section above. All sample types seem to have produced similar results as far as data quality is concerned. We will move forward with confirmation and further experimentation in the pyramidal neuron population from the same brain areas. This decision is based on the data reported above that indicates differences for multiple levels of transcriptional regulations in this cell population between control and ASD samples.

**Actual or anticipated problems or delays and actions or plans to resolve them**

There were delays outlined in the 2013 annual report for this project regarding the laser capture microdissection instrument. That instrument has since been replaced giving our lab continuous access to this machine. Overall, these delays did push back the initial start of this project. We are now working in an approved no-cost extension (NCE) period of the project. A revised SOW with new projected target dates for the remaining work was submitted in the NCE request. We fully anticipate being able to complete the project based on the new timeline.

## PRODUCTS

**Books or other non-periodical, one-time publications:**

Crawford, Jessica D. "Cellular-based Brain Pathology in the Anterior Cingulate Cortex of Males with Autism Spectrum Disorder" December 2014. Dissertation. Accepted. (Withheld from publication for 1 year)

**Other Products:**

Whole transcriptome sequencing data was produced for 25+ samples. These samples represent different tissue/cell types as well as different subject demographics. This information will be release to an autism database following a full analysis by our lab.

## PARTICIPANTS & OTHER COLLABORATING ORGANIZATION

**What individuals have worked on the project?**

| | |
|---|---|
| Name: | Gregory A. Ordway, Ph.D. (No Change) |

| | |
|---|---|
| Name: | Michelle J. Chandley, Ph.D. (No Change) |

| | |
|---|---|
| Name: | Jessica D. Crawford, Ph.D. |
| Project Role: | Trainee |
| Research Identifier: | |
| Nearest person month worked: | 12 |
| Contribution to Project: | Responsible to the molecular biology aspects of the project. |
| Funding Support: | Graduate Assistantship/Department Funds (in-kind) |

**Has there been a change in active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

**Ongoing Research Support**

**The following grants have ended for the PI:**

5R01 MH 46692 Ordway (PI) 09/01/91-03/31/13
NIMH "Noradrenergic System in Depression"
This research tests the hypothesis that dysfunction of noradrenergic neurons is closely associated with glial disruption in major depressive disorder. Laser capture microdissection and quantitative PCR methods are employed to study gene expression along specific pathways in noradrenergic locus coeruleus neurons, astrocytes and oligodendrocytes from assiduously matched control and major depressive disorder subjects.

Distinguished Investigator Award Ordway (PI) 2/9/09-2/8/11
Amer. Found. for Suicide Prev. "Glutamatergic signaling in the locus coeruleus in depression and suicide"
The major goal to examine the quantitative expression of glutamate receptor genes in noradrenergic neurons in the human locus coeruleus from victims of suicide that had major depression at the time of death, and to determine whether glutamate receptor gene expression changes are also observed in the entorrhinal cortex.

AS#7330 Ordway (PI)
Autism Speaks "Glia Pathology in Autism" 3/1/11-2/28/13
The goal of this project is to measure levels of expression of several genes associated with glutamate transmission in pyramidal neurons and surrounding astrocytes in postmortem anterior cingulate cortex of young adult autism subjects and matched normal control subjects.

**The following grant has been awarded to the PI:**

SRG-0-100-13 Ordway (PI)
American Foundation for Suicide Prevention; "Oxidative DNA Damage in Brainstem Oligodendrocytes in Depressed Suicide Victims; 6-3-14 to 7-1-16
This project examines whether the relative density of noradrenergic innervation to a brain region affects the susceptibility of oligodendrocytes to telomere shortening and oxidative stress as observed in depressed suicide victims. To examine this, oligodendrocytes will be captured from the region of the brainstem locus coeruleus (high norepinephrine) and occipital cortex white matter (low norepinephrine) from depressed suicide victims and matched psychiatrically normal control subjects.

**What other organizations were involved as partners?**

Nothing to report.

## SPECIAL REPORTING REQUIREMENTS

Nothing to report.

## APPENDICES

REFERENCES


1. Chandley, M. J. *et al.* Gene expression deficits in pontine locus coeruleus astrocytes in men with major depressive disorder. *J. psychiatry Neurosci. JPN* **38,** 120110 (2012).

2. Ordway, G., Szebeni, A., Duffourc, M. M., Dessus-Babus, S. & Szebeni, K. Gene Expression Analyses of Neurons, Astrocytes, and Oligodendrocytes Isolated by Laser Capture Microdissection From Human Brain: Detrimental Effects of Laboratory Humidity. *J. Neurosci. Res.* **87,** 2430–2438 (2009).

3. Baggerly, K. A., Deng, L., Morris, J. S. & Aldaz, C. M. Differential expression in SAGE: accounting for normal between-library variation. *Bioinformatics* **19,** 1477–83 (2003).

4. Xiang, L. *et al.* Dopamine receptor gene expression in human amygdaloid nuclei: elevated D4 receptor mRNA in major depression. *Brain Res.* **1207,** 214–24 (2008).

5. Ewing, B. & Green, P. Base-Calling of Automated Sequencer Traces Using Phred. II. Error Probabilities. *Genome Res.* **8,** 186–194 (1998).

6. Ewing, B., Hillier, L., Wendl, M. C. & Green, P. Base-Calling of Automated Sequencer Traces Using Phred. I. Accuracy Assessment. *Genome Res.* **8,** 175–185 (1998).

7. Ameur, A. *et al.* Total RNA sequencing reveals nascent transcription and widespread co-transcriptional splicing in the human brain. *Nat. Struct. Mol. Biol.* **18,** 1435–40 (2011).

8. Kapranov, P. *et al.* The majority of total nuclear-encoded non-ribosomal RNA in a human cell is "dark matter" un-annotated RNA. *BMC Biol.* **8,** 149 (2010).

9. Ginsberg, S. D., Alldred, M. J. & Che, S. Gene expression levels assessed by CA1 pyramidal neuron and regional hippocampal dissections in Alzheimer's disease. *Neurobiol. Dis.* 1–9 (2011). doi:10.1016/j.nbd.2011.07.013

10. Wilhelm, B. T. & Landry, J.-R. RNA-Seq-quantitative measurement of expression through massively parallel RNA-sequencing. *Methods* **48,** 249–57 (2009).

11. Wang, Z., Gerstein, M. & Snyder, M. RNA-Seq: a revolutionary tool for transcriptomics. *Nat. Rev. Genet.* **10,** 57–63 (2009).

12. Nagalakshmi, U., Waern, K. & Snyder, M. RNA-Seq: a method for comprehensive transcriptome analysis. *Curr. Protoc. Mol. Biol.* **Chapter 4,** Unit 4.11.1–13 (2010).